AN ESTIMATE OF THE NUMBER AND SIZE OF DNA REPLICATING UNITS IN CULTURED HUMAN (HeLa S3) CELLS

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Running Head: HeLa DNA replicating units

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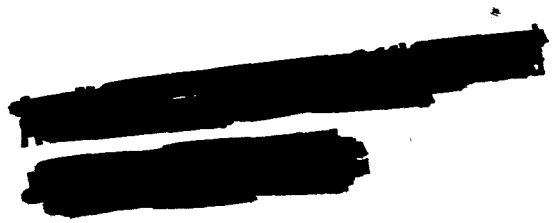
ABSTRACT

With the use of the equilibrium density gradient centrifuge technique with tritium and C^{14} labeled DNA precursors, a means for estimating the numbers of sites per HeLa S3 cell synthesizing DNA was developed. In unsynchronized cultures, there are approximately 3 to 4×10^3 such sites per cell. These data were used to show that the upper limit for the average molecular weight of DNA of the HeLa S3 replicating unit is about 3×10^9.



SUMMARY

The DNA replication of HeLa S3 cells was examined, using the equilibrium density gradient centrifuge technique with C^{14} bromouracil deoxyriboside (C^{14}BUdR) to form and detect the DNA of hybrid density (one light - one heavy strand). When the cells were prelabeled for one generation time, (24 hours) or more with H^3 thymidine followed by a 2- or 3-hour incubation with C^{14}BUdR, the results showed, as expected, the presence of the hybrid band, as indicated by the C^{14} counts, and the normal band, as indicated by the o.d. 260 mμ readings and the bulk of the tritium counts. Coincidentally with the C^{14} peak was a small tritium peak indicative of the fraction of the prelabeled DNA that was hybridized. When the C^{14}BUdR incubation (2-3 hours) was preceded by a pulse label with H^3 thymidine of 30 minutes or less, 30% or more of the tritium counts appeared at densities intermediate between the hybrid (C^{14}) and normal (o.d. 260 mμ) peaks, with the remainder at densities representing normal DNA. It was shown that this distribution was not due to carryover from a pool of tritiated precursors of DNA, but to an end-to-end association of tritium and density (C^{14}BUdR) label. This finding was used to estimate that the average number of replicating sites per cell is $3\text{-}4\times10^3$. This figure can be used to show that the upper limit of the average molecular weight of the DNA in the replicating unit of HeLa S3 is about 3×10^9; it may be lower than this figure if the number of sites replicating DNA during any one 30-minute period is considerably fewer than the total number of replicating units in the cell.

1. INTRODUCTION

Evidence indicates that in bacteria, there is generally only one growing point per chromosome (Nagata, 1963; Yoshikawa and Sueoka, 1963; Bonhoeffer and Gierer, 1963) although Yoshikawa, O'Sullivan and Sueoka (1964) have presented data indicating that, under rapid growing conditions, the chromosome of Bacillus subtilis may begin a second replication before the first one is completed. The situation in higher organisms is obviously more complicated. In all mammalian cells there are several chromosomes, and it seems likely that each chromosome must have a minimum of one replication unit. Actually, the evidence is for even more complexity than this. Autoradiographic studies in a number of mammalian cell types (Taylor, 1960; Painter, 1961; Stubblefield and Mueller, 1962; Moorhead and Defendi, 1963) have shown that many of the chromosomes have two or more sites undergoing DNA replication at the same time that other parts of the same chromosomes are not replicating DNA. It does not appear possible however, to determine more precisely the number of DNA replicating sites using autoradiography, because of limitations in resolution of this technique.

In the course of experiments on cultured mammalian cells using the equilibrium density gradient technique reported in this paper, we developed a method to estimate the average number of sites replicating DNA at any one time in mammalian cells. This method resulted from modifications of the experiments of Lark, Repko and Hoffman (1963) and Bonhoeffer and Gierer (1963), that have suggested only one growing point per chromosome is present in Escherichia coli. The rationale of the procedure is that after a short pulse with a radioactive label, followed by a density label, the association of

radioactive label with density label will be small, if during isolation of the DNA shearing creates pieces (break sizes) very much smaller than the size of the labeled portion of the chromosome, so that only an extremely small fraction of the pieces have an end-to-end association of tritium with the density label. On the other hand, the association will be relatively large if the break sizes are of the same size or larger than the labeled size. In our studies with mammalian cells, we found that following a $H^3TdR^\dagger$ pulse of one-tenth or less of the average time required for the cell to completely replicate all its DNA (S time), a significant fraction of the tritium occurred in association with the density label, or at densities intermediate between the hybrid and normal peaks. After establishing that this was not due to carryover of H^3TdR in the intracellular pool, we have exploited this finding to estimate the average size of the piece labeled during the pulse, and consequently, about how many such pieces must be present in the cell during the labeling period.

2. METHODS AND MATERIALS

(a) Cell lines and media

HeLa S3 monolayer cell cultures were used throughout the study. In most cases they were grown in plastic petri dishes in water-saturated air containing 5% CO_2. In a few cases the cells were grown in the same atmosphere in rubber-stoppered culture flasks. The medium at all times was Eagles (Microbiological Associates) supplemented with 15% fetal calf serum. All incubations were at 37°C.

than unity for Q_1a and Q_1b, because of the low transconductance achieved with MOS devices at low current levels. Secondly, it is possible to adjust the drain current so that the temperature coefficient of the gate-to-source voltage is approximately zero. This is done by adjusting R_1 and R_6 to allow for drain currents of approximately 250 μA. R_7 and R_8 form a 2 to 1 negative feedback potential divider, which sets the closed-loop gain at 2. The feedback capacitor value can be calculated from equation (3) if the maximum shunt capacity at the input is assumed to be 10 pf. Rearranging equation (3) yields

$$C_f = \frac{C_t}{K - 1} = \frac{10}{2 - 1} = 10 \text{ pf}$$

Care must be taken to insure the stability of a circuit which has a high open-loop gain with both overall positive and negative feedback. In the present design, resistor R_2 (22K) is shunted with C_1 (470 pf), giving the 20 db/decade roll-off in open-loop frequency response necessary for stability.

If the negative capacity control, R_{11}, is overadjusted the circuit will oscillate, but when the circuit is adjusted for critical damping, it is stable.

Performance

The measured performance of the negative capacity preamplifier is tabulated below:

Voltage gain	2
Bandwidth - 50 Ω source	0 - 140 kcps

Input time constant with compensation adjusted for critical damping with:	
(a) 22 MΩ source	9 μs
(b) 100 MΩ source	12 μs
Input resistance	$> 10^{14}$ Ω
Drift measured at preamplifier output	75 μV/°C
Input leakage current	0.9×10^{-15} A
Equivalent input noise voltage*	
(a) With a 22 MΩ source resistance, measured over a 3 db bandwidth of 17.8 kcps, corresponding to a 9 μs input time constant.	198 μV RMS
(b) With a 100 MΩ source resistance, measured over a 3 db bandwidth of 13.3 kcps, corresponding to a 12 μs input time constant.	234 μV RMS
Power required	±9.4 V at 2.4 mA
Weight	4 g
Volume	3.6 × 1.1 × 1.0 cm

*A 22 MΩ resistor generates 104 μV RMS over a 3 db bandwidth of 17.8 kc, while a 100 MΩ resistor generates 187 μV RMS over a 3 db bandwidth of 13.3 kc.

Figure 5 shows the response of the circuit to a square wave input with a 22 MΩ source impedance. The square wave cannot be applied from a voltage source through a resistor because the shunt capacity of the resistor causes a peaking effect which results in an apparent rise

The contents of the dialysis bag were gently rocked with 0.07 ml 10% SDS and an equal volume of freshly redistilled phenol equilibrated with NaCl 0.1 M, EDTA 0.01 M, Tris buffer 0.01 M, centrifuged, treated 4 more times with this mixture and 1 time with chloroform. The sample was then dialyzed overnight against two 500 ml changes of NaCl 0.1 M, EDTA 0.001 M, Tris buffer 0.0001 M. The final sample had a volume of about 1 ml and an o.d. at 260 mμ of 0.337 and at 280 mμ of 0.240. Ten μl yielded 1.15×10^4 cpm tritium at a counting efficiency of 23%.

For the determination of molecular weight of the DNA as isolated by the lysis method, HeLa cells were grown in C^{14}TdR (30 mc/mM) for 48 hours and lysed as previously described for CsCl density gradient work. The lysate was divided into three parts. One was handled in the usual way with no further treatment after lysis. The second was deproteinized by mixing on a vortex mixer with an equal volume of chloroform-amyl alcohol (24:1). The third was similarly deproteinized and then subjected to vigorous fragmentation by stirring for 2 minutes at high speed with an Omnimixer (Ivan Sorvall, Inc.). Each sample was then diluted, CsCl added, centrifuged at 37,000 rpm for 48 hours, and 2-drop fractions collected as described before. The fractions containing the highest counts, in each case, were pooled and dialyzed overnight against NaCl 0.1 M, EDTA 0.001 M, Tris 0.001 M. The dialysates were then concentrated by evaporation with an air stream to about 0.5 ml, and 10 μl of each sample layered over a 5-20% sucrose gradient in a 5 ml centrifuge tube. In each case the 10 μl contained less than 0.3 μg DNA. On each tube was also layered 5 μl of the phage T7 H^3DNA and the tubes were centrifuged at 28,000 rpm for 4 hours at 4° C. Two-drop fractions were collected directly into scintillation counting vials containing the counting fluid, and the radioactivity determined.

(e) Radioactivity determinations

All radioactivity determinations were performed with a Packard Tri-carb Liquid Scintillation Spectrometer. The aqueous samples were taken into a scintillation counting mixture previously described (Painter and Rasmussen, 1964). The channel gains were so arranged that the tritium contributed no significant numbers of counts to the channel used for counting C^{14}. The C^{14} contribution to the channel used for tritium was determined by freshly prepared standards. The tritium and C^{14} counts per sample could then be computed without resort to simultaneous equations.

3. RESULTS

When a HeLa S3 culture is grown for one generation time (24 hours) or more in the presence of low specific activity H^3TdR (1 µg/ml; 30-60 mc/mM), washed and shifted to C^{14}BUdR medium (5 µg/ml) for 2 or 3 hours, and the whole lysate analyzed in the equilibrium density gradient, the pattern shown in Fig. 1 is observed. A prominent hybrid density peak is formed by C^{14} counts accompanied by a small tritium peak, indicating the fraction of prelabeled DNA hybridized. The density shift is about 16 "normalized" drops (all patterns are normalized to 100 total drops) which indicate a substitution of BU for thymine of about 30%. These results are in reasonable agreement with those of Simon (1961, 1963), who studied the replication of HeLa S3 DNA using the analytical ultracentrifuge. Both the hybrid and normal peaks (the latter measured either by tritium or o.d. at 260 mµ) are skewed quite considerably to the light side, and accompanying the tailing in the light portion of the normal peak is a secondary peak of C^{14} counts. This peak always occurs in unsheared lysates, and although it was at first attractive to think that it might be of biological importance, the evidence now indicates that it is probably artifactual in nature.

Fig. 1

When such a peak was rebanded using a HeLa DNA unlabeled marker, the great majority of the C^{14} counts banded at the hybrid density (e.g., see Fig. 5). However, a small fraction still appeared in the light "tail", and it could be hypothesized that the drop-collection procedure in the first run had somehow disrupted a complex between most of the DNA and some lighter material (protein?) that caused the DNA to appear at a less dense position. The experiment that appears to prove that this result is artifactual, however, was done as follows: A lysate of cells labeled with H^3TdR and then with C^{14}BUdR was subjected to shear by exposing to ultrasound for 1 minute (Branson Instruments, Inc.) and run in the density gradient. In this case, no C^{14}BUdR counts were found at the light end of the normal peak (Fig. 2(a)). Upon rebanding of the hybrid band, along with a purified, but unsheared, HeLa DNA marker, a secondary C^{14} peak reappeared in the light tail of this marker (Fig. 2(b)). This result demonstrates that the high molecular weight normal DNA in some manner displaces the last portion of the hybrid DNA before the latter comes out of the centrifuge tube. The normal DNA is apparently so viscous that it acts almost as a single large entity and nearly completely empties before the remainder of the hybrid can do so.

Figs. 2(a) and 2(b)

A series of experiments were performed in which the cells were pulse-labeled for 30 minutes with H^3TdR, washed, incubated with C^{14}BUdR, lysed and analyzed by the density gradient technique. Figure 3 demonstrates the distribution of tritium and C^{14} following the prior 30-minute pulse with H^3TdR. A significant portion of the tritium counts lies to the hybrid side of the normal peak, even at the short incubation times. At 18 hours a considerable portion of the tritium is in the hybrid area, but this can be explained on the basis of some faster growing cells having gone through a second replication and thus having truly hybridized the tritium with the

Fig. 3

density label (H^3TdR label on one strand, C^{14}BUdR on the other). At 48 hours all the tritium had hybridized, but, at least in this instance, a heavy (two C^{14}BUdR strands) band did not appear.

The appearance of tritium to the heavy side of the normal peak at 1-1/2 hours cannot be explained on the basis of hybridization, since the generation time of the cell is about 24 hours and no DNA would go through a second replication this quickly. The semiconservative model for DNA replication appears to hold for HeLa S3 cells (Simon, 1961) and only two possibilities appear to be capable of explaining this observation. One is that H^3TdR that has been incorporated into the precursors of DNA continues to label the DNA after washing by being incorporated into DNA along with the C^{14}BUdR. The second possibility is that after serially labeling with H^3TdR and C^{14}BUdR, and isolation of DNA, a relatively high fraction of the broken pieces of DNA contain an end-to-end association of tritium with the C^{14} density label, so that measurable amounts of tritium label appear in hybrid and intermediate densities.

Several lines of evidence appear to indicate the former possibility to be, at most, a minor contributor to the tritium found at densities heavier than the normal DNA. First, the specific activity of DNA of HeLa S3 cells that have been pulsed with H^3TdR and then grown in unlabeled media, with or without a carrier, does not increase over that of DNA from HeLa S3 cells pulsed with H^3TdR and fixed immediately. Table 1 shows the total DNA tritium counts and specific activity of HeLa S3 cells that have been pulsed for 15 minutes with tritium, washed 2 times, and either fixed immediately or incubated for 10 minutes in nonradioactive medium before fixing. There is no significant difference in the total tritium counts in the two kinds of

Table 1

incubations, and the specific activities of the cultures that were incubated for 10 minutes in unlabeled medium are slightly lower than the cultures fixed immediately. Table 2 shows the results of experiments where 12 cultures were pulsed with H^3TdR for 30 minutes and the cells washed 3 times. Table 2

Four cultures were analyzed immediately. The medium of four plates was replaced with a nonradioactive Eagles medium containing 5 μg/ml BUdR. The medium on the other four was replaced with unsupplemented Eagles medium, and all 8 cultures were incubated for another hour before analysis for DNA specific activity. The results show that the DNA specific activity of the cells grown one additional hour in BUdR-supplemented medium is lower than that of DNA of cells fixed immediately after the H^3TdR pulse. Since additional growth in the absence of radioactivity results in lower specific activity, this result indicates that no significant incorporation of H^3TdR into DNA occurred after the medium change. The results with cells grown in unlabeled, unsupplemented Eagles medium are not significantly different from cells fixed immediately after pulsing. However, the data show that no incorporation of the order of 20-30% (the fraction found outside the normal peak in the density gradient experiments) could have occurred.

A second type of evidence that shows the association of tritium with density label is end-to-end, rather than mixed, is the effect of shearing on the amount of tritium appearing in the heavy side of the normal peak. If the association is end-to-end, shearing should reduce the amount of tritium in hybrid and intermediate densities, but if the tritium is incorporated along with the density label, shearing should have no effect. Pettijohn and Hanawalt (1964) used this principle to demonstrate that intermediate density DNA they isolated from E. coli was actually due to recovery of points of transition from light to hybrid.

In an experiment to test these alternatives, a HeLa S3 lysate that had been pulsed with H^3TdR for 50 minutes followed by C^{14}BUdR was added to CsCl and divided into two parts. One was not further treated, but the second was fragmented by one minute of sonication. More than 30% of the tritium in the untreated lysate (Fig. 4) as found at densities greater than normal (260 mμ o.d. peak), while much less ($\leq$ 13%) was found there in the fragmented sample (Fig. 2(a)).

Fig. 4

The exact measurement of radioactivity in fractions at intermediate and hybrid densities is difficult because at densities very close to the normal density peak, the tritium or carbon counts may arise from DNA of either intermediate or normal density. However, the optical density of the normal DNA peak in undisturbed lysates rises very rapidly as fractions are collected, generally ascending from the background reading to the peak reading in 4 or fewer drops, and never exceeding 6 drops. When the radioactivity of fractions collected only at two drops or more to the heavy side of the onset of the optical density (normal DNA) peak is assigned to intermediate density, the estimation of radioactivity in hybrid and intermediate density fractions is probably always somewhat smaller than the real value. The extent of this underestimate is relatively small however, since the tritium content of fractions closer to the normal bands generally is less than that at densities a few drops further to the heavy side. The final estimate of total radioactivity in heavier-than-normal densities also must take into account the material found in fractions of the "secondary peak" at the light side of the normal peak. As discussed above, when C^{14}BUdR is used, all C^{14} counts found in these fractions are actually from hybrid or intermediate density DNA. A certain amount of the tritium is also of this kind (Fig. 5(a)), and only rebanding of this portion can yield a reasonable estimate of the amount of

Figs. 5(a) and 5(b)

tritium of heavier-than-normal DNA that is in these fractions (Fig. 5(b)). Again, however, this is generally a small percentage of the total intermediate density radioactivity, and only in a few cases has it contributed enough to appreciably change the estimate of the fraction of radioactivity found at heavier-than-normal DNA densities.

Experiments were performed in which the tritium pulse was followed by an incubation in unlabeled medium before addition of C^{14}BUdR. In one experiment, the results of which are illustrated in Fig. 6, the H^3TdR pulse was 15 minutes, followed by washing and incubation in unlabeled medium for 10 minutes before the addition of 5 μg/ml C^{14}BUdR. Again, in the untreated lysate (Fig. 6(a)) tritium was found at heavier-than-normal densities, but the amount is only about 20% of the total tritium in the gradient. In the lysate that was treated with a procedure similar to that used for deproteinization, the amount of tritium found outside the normal peak was less than 13% (Fig. 6(b)), and in the lysate sheared with the Omnimixer, the density label-associated tritium was less than 5% (Fig. 6(c)). The latter figure is difficult to determine because one effect of shear is to increase the peak widths to such an extent that the hybrid and normal peaks overlap considerably. The 5% figure represents the upper limit of tritium found outside the normal peak that can be attributed to "pool" tritium incorporated simultaneously with the C^{14} density label, and is actually probably no more than 2%.

Figs. 6(a), 6(b), and 6(c)

Figure 7 shows the results of rebanding portions of the centrifuge runs of Fig. 6. Rebanding of the area of hybrid and intermediate density of the untreated lysate (7(a)) yields a well-formed peak 4 drops to the heavy side of the normal HeLa marker DNA, while rebanding of a similar portion of the "deproteinized" lysate yields a peak only two drops to the left of the marker (Fig. 7(b)). These rebands show that the intermediate density

Figs. 7(a) and 7(b)

tritium peaks are real, and indicate the effect of shaking is to reduce their density by decreasing the amount of density label associated with the tritium.

An experiment was performed in which a 30-minute pulse with H^3TdR was followed by a 30-minute pulse with C^{14}TdR, and finally incubation with unlabeled BUdR for 3 hours. In this experiment the C^{14} label would be expected to appear at about the same intermediate densities that tritium did in previous experiments, and as Fig. 8 illustrates, it did so. On the other hand, the tritium label assumes a pattern almost coincidental with that of the 260 mμ o.d. measurements, indicating that one-half hour between a pulse and density label results in almost no end-to-end association of the two labels. A small tritium tail to the heavy side of the normal peak suggests that a small amount of association occurs, probably the result of a few large molecules with tritium at one end and a small amount of BU at the other.

Fig. 8

The average molecular weight of the DNA isolated by the detergent freeze-thaw method was determined with the sucrose gradient as described in Methods. The results on an undisturbed lysate, a deproteinized lysate, and a lysate fragmented by 2-minute treatment with the Omnimixer are illustrated in Fig. 9. The average molecular weights calculated according to the equation of Burgi and Hershey (1963) are 118, 71, and 6.6×10^6, respectively, using 27×10^6 as the molecular weight of phage T7 DNA (Burgi and Hershey, 1963, based on the results of Davison and Freifelder, 1962). The effect of the deproteinization method thus appears to be to reduce the average "break size" of isolated DNA by about 40%, and the effect of shearing by the Omnimixer is such that the molecular weight is reduced to about 1/20 of that in the undisturbed lysate.

Figs. 9(a) and 9(b)

4. DISCUSSION

That there is an end-to-end association of tritium with a C^{14}BUdR density label after short pulses of H^3TdR suggests that this finding can be used to estimate the average number of replicating sites per HeLa cell nucleus. Consider the situation where the "break size" is very much smaller than the labeled size, such as has been the case in some bacterial experiments (Lark et al., 1963; Pettijohn and Hanawalt, 1964; Hanawalt and Ray, 1964). The fraction of pieces containing both tritium and the hybrid label is only of the order of 1 per 100, and thus, the amount of tritium to the heavy side of the normal DNA density is essentially immeasurable. The other extreme is (a hypothetical) one where the break size is very much larger than the labeled piece. In such an instance the broken piece would, in all but a few rare cases where the break occurred within the labeled piece, include both tritium and density labels, and so practically all the tritium would be associated with the density label, i.e., at densities greater than normal DNA.

In intermediate cases, the fraction of tritium label appearing at densities greater than the density of normal DNA (F) will depend on just two parameters: the size of the labeled piece of DNA (L), and the "break size" (B). In fact, it can easily be shown that:

$$F = \frac{B}{L + B} \qquad (1)$$

Experimentally, one can estimate both F, by measuring the amount of tritium to the heavy side of the normal peak in the density gradient and dividing by the total amount of tritium in the gradient; and B, by determining the average molecular weight of DNA. The average molecular weight of the labeled piece can then be estimated by rearranging equation (1) to:

$$L = B\left(\frac{1}{F} - 1\right) \qquad (2)$$

In the case of the undisturbed lysate of HeLa cells (Fig. 4) where $B \simeq 1.2\times10^8$ and with a 30-minute incubation, $F \simeq 0.33$, so that $L \simeq 24\times10^8$. Now, 30 minutes is approximately one-twelfth the DNA synthesis time (S period) of HeLa cells (Painter and Drew, 1959) and so on the average, the HeLa cell will synthesize about one-twelfth of its DNA in this time. The DNA content of the HeLa cell is 17 picograms, or a total molecular weight of 10^{13} (Lee and Puck, 1960), so that the total DNA synthesized per average HeLa cell in a 30-minute incubation corresponds to a molecular weight of about 8×10^{11}. Since the average size of the labeled piece of DNA (L) synthesized in this time is 2.4×10^8, there must be $(8\times10^{11}/2.4\times10^8) \simeq 3.3\times10^3$ such pieces per cell.

In the case of cells pulsed with H^3TdR, followed by incubation with no label before final density labeling, the fraction (F) of tritium found associated with the density label depends on three factors; B and L, as before, and U, the size of the unlabeled piece. It can be shown that:

$$F = \frac{B - U}{B + L} \qquad (3)$$

Now since the ratio U/L can be estimated simply by dividing the time of incubation with no label by time of tritium labeling, this can be substituted into the equation and rearranged to:

$$L = \frac{B(1 - F)}{F + \alpha} \qquad (4)$$

where $\alpha = U/L$, estimated by incubation times. In the experiment where this procedure was used (Fig. 6), F is for the undisturbed lysate $\simeq 0.2$, and $\alpha = 2/3$ (a 10-minute "unlabeled pulse" and a 15-minute H^3TdR pulse), so that L

solves to 1.1×10^8. Since the H^3TdR pulse time in this experiment was one-half of that in the experiment where the H^3TdR pulse was followed immediately by the density label, the figures are in excellent agreement.

The estimate of $3\text{-}4 \times 10^3$ replicating sites per cell is obviously an average figure derived from an asynchronous culture. To estimate with confidence the average molecular weights of these sites, the average fraction of total replicating sites in operation at any one time must be known, and it is not. Some speculation on this figure can be made, however. In the HeLa S3 culture, asynchronous replication of the chromosomes has been reported (Painter, 1961; Stubblefield and Mueller, 1962). However, the cell exhibiting asynchronous chromosomal replication is the exception rather than the rule. Table 3 shows the results of an experiment where cultures were pulse labeled with H^3TdR, serially treated with colchicine and hypotonic salt solution at various times thereafter, and autoradiograms prepared to determine the extent of asynchrony in the population of cells. These results show that the greatest percentages of incompletely labeled metaphases appear in the first few hours after the pulse, and that at later times the metaphases become much more uniformly labeled. In the culture treated from hour 6 to hour 27 with colchicine, 90% of the labeled metaphases showed uniform labeling. Since the labeled metaphases seen earliest after the pulse were the closest to end of the S phase at the time, these results indicate that asynchronous replication occurs predominantly near the end of S. These results suggest that during most of the S period, all of the chromosomes are replicating their DNA at close to the maximum rate. Although other models can be suggested, this can be interpreted to indicate that during most of the S period, practically all the replicating sites in the cell are operating, and only near the end of S do they "shut off" differentially. That one of the X chromosomes

Table 3

of female mammals begins and ends DNA replication at later times than do the autosomes is now well known (Taylor, 1960; Morishima, Grumbach, and Taylor, 1962), but even here it is probable that during much of S time DNA replication is occurring in all the chromosomes.

If the number of replicating sites determined by the density gradient method is practically equal to the total number of replicating units that occur in a cell, the average molecular weight of each replicating unit in the HeLa S3 cell can be estimated to be about $10^{13}/3.3\times10^{3} \simeq 3\times10^{9}$. This figure is attractive because it is close to the molecular weight of the DNA of a single "replicon" in E. coli (Cairns, 1963). If, on the other hand, the number of replicating sites determined detects only a small fraction of the total number of replicating units, because only this fraction is in active replication during the tritium labeling period, the average molecular weight per replicating unit would be smaller than 3×10^{9}. In any case the rate of DNA synthesis along each replicating unit is considerably slower than along the chromosome of E. coli (a maximum of about 10^{7} daltons DNA per minute for HeLa compared to about 10^{8}/minute for E. coli), even though the HeLa cell synthesizes more total DNA per unit time.

Under any circumstance, these experiments set an upper limit on the size of the replicating unit in HeLa S3 cells. On the basis of the effect of X-radiation on the rate of DNA synthesis in HeLa S3 cells, we previously suggested that there existed DNA replicating units of the order 10^{9}-10^{10} daltons in these cells (Painter and Rasmussen, 1964). The good agreement between that estimate and the one arrived at in this work strongly suggests that the DNA replicating system in HeLa cells is made up of units of about this size, and would indicate that the chromosomes contain 10-100 such units

each. How these are organized spatially and how they are associated with chromosomal protein remains obscure. However, the requirement for (at least) this many units per chromosome, combined with the asynchrony of replication observable within single chromosomes, restricts the kinds of chromosomal models that can be constructed.

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TABLE 1

A comparison of tritium counts and specific activity in DNA of cultures pulsed for 15 minutes with H^3TdR, washed, and either fixed immediately or incubated 10 minutes with a nonradioactive medium before fixing

Incubation type	o.d. at 260 mμ	DNA fraction	
		cpm H^3 per ml sample	Specific activity cpm/μg DNA×10^{-3}
H^3 15 min, immediate fix	0.488	27654	1.19
H^3 15 min, immediate fix	0.470	27558	1.23
H^3 15 min, 10 min addit.	0.520	27730	1.12
H^3 15 min, 10 min addit.	0.533	27706	1.09

TABLE 2

Specific activities of HeLa S3 DNA after incubation with H^3TdR for 30 minutes, washing, and either (1) fixing immediately, or (2) incubating for one additional hour with medium containing 5 μg/ml BUdR, or (3) incubating one additional hour with unsupplemented medium

Sample type	Specific activity cpm/μg DNA×10^{-3}	Average specific activity
Immediate fixing	3.75	3.56
	3.59	
	3.70	
	3.20	
BUdR medium 1 hour	3.30	3.26
	3.29	
	3.27	
	3.29	
Medium 1 hour	3.55	3.43
	3.85	
	3.51	
	3.25	

TABLE 3

Distribution of partly and completely labeled mitotic figures among HeLa S3 cells in cell division at intervals after 15-minute incubation with 1 μc/ml H^3TdR

Hour†	Mitotic index	Total mitotic cells counted	Partially		Completely		% of labeled mitoses that are completely labeled
			Number	%	Number	%	
0	16.5	70	0	(0)	0	(0)	-
1	7.3	67	0	(0)	0	(0)	-
2	10.0	95	5	(5.3)	0	(0)	0
3	7.0	200	29	(14.5)	6	(3.0)	17
4	19.0	279	99	(35.5)	64	(22.9)	39
5	24.0	200	38	(19.0)	111	(55.5)	75
6	21.0	254	59	(23.2)	126	(49.6)	68
6-27	159.0	344	13	(3.8)	181	(52.6)	93

†Time of addition of colchicine for 1-hour incubation, except for last entry, in which colchicine incubated with cells from the 6th through the 27th hour.

FIGURE LEGENDS

Fig. 1. Density gradient profile of DNA of HeLa S3 labeled for 30 hours with H^3TdR before washing and incubation with C^{14}BUdR for 2 hours. Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity.

Fig. 2(a). Density gradient profile of DNA of HeLa S3 labeled for 30 minutes with H^3TdR before washing and incubation with C^{14}BUdR for 30 hours. The lysate in CsCl was subjected to shearing by ultrasonic radiation for 1 minute before centrifuging. Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity; ⊙ ——— ⊙ optical density at 260 mμ.

(b) Density gradient of material forming the C^{14} peak of Fig. 2(a) with unlabeled normal HeLa S3 DNA added as marker of normal DNA position. Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity; ⊙ ——— ⊙ optical density at 260 mμ (HeLa DNA marker).

Fig. 3. Density gradient profiles of DNA of HeLa S3 labeled for 30 minutes with H^3TdR before washing and incubation with C^{14}BUdR for various lengths of time: (a) 1-1/2 hours with C^{14}BUdR; (b) 18 hours with C^{14}BUdR; (c) 44 hours with C^{14}BUdR; Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity; O ——— O o.d. at 260 mμ.

Fig. 4. Density gradient profile of DNA of HeLa S3 pulse labeled with H^3TdR for 30 minutes before washing and incubation with C^{14}BUdR for 3 hours. Lysate was mixed with CsCl and total mixture divided into 2 parts. One part was centrifuged without further treatment and the data of this figure represent the results of that run; the other part was sonicated before centrifuging, and the results are presented in Fig. 2(a).

Fig. 5(a). Density gradient profile of DNA of HeLa S3 pulse labeled for 10 minutes before washing and incubation with C^{14}BUdR for 2 hours. Note marked C^{14} peak at light end of normal density, and large skewing right of tritium activity. Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity; ⊙ ——— ⊙ o.d. at 260 mμ. (b) Reband of radioactivity from light density portion of normal DNA of Fig. 5(a). Almost all the H^3 activity peaks at a density between C^{14} and added HeLa DNA marker, as represented by o.d. 260 curve. Symbols same as Fig. 5(a).

Fig. 6. Density gradient profiles of DNA of HeLa S3 labeled with H^3TdR for 15 minutes, washed, and then incubated in the absence of any label for 10 minutes before the final incubation for 2 hours with C^{14}BUdR. (a) Undisturbed lysate; (b) Lysate shaken on vortex mixer for one minute, in a manner used for deproteinization; (c) Lysate stirred violently for 2 minutes with Omnimixer. Δ ——— Δ C^{14} activity; □ ——— □ tritium activity; O – – – O optical density at 260 mμ.

Fig. 7. Density gradient profiles of hybrid and intermediate density areas of Fig. 6. (a) Reband of fractions to the heavy side of normal density band of Fig. 6(a). Δ ——— Δ tritium activity; □ ——— □ C^{14} activity; O ——— O o.d. of added HeLa S3 DNA marker. (b) Reband of fractions to the heavy side of normal density band of Fig. 6(b). Symbols same as for Fig. 7(a).

Fig. 8. Density gradient profile of DNA of HeLa S3 that had been labeled for 30 minutes with H^3TdR, washed, labeled 30 more minutes with C^{14}TdR, before a final incubation for 2 hours with nonradioactive BUdR. Δ ——— Δ tritium activity; ⊡ ——— ⊡ C^{14} activity; ⊙ ——— ⊙ o.d. at 260 mμ.

Fig. 9. Sucrose gradient (5-20%, 4 hours) profiles of DNA of HeLa S3, labeled with C^{14}TdR, and centrifuged along with tritium labeled bacteriophage T7 DNA. (a) Undisturbed lysate; (b) lysate shaken on vortex mixer; (c) lysate stirred violently with Omnimixer for 2 minutes. Δ ——— Δ tritium activity (phage); ⊡ ——— ⊡ C^{14} activity (HeLa).

FOOTNOTE

†Abbreviations used: TdR, thymidine; BUdR, 5-bromouracil deoxyriboside; EDTA, ethylene dianine tetraacetic acid; Tris, 2 amino-2(hydroxymethyl)-1,3 propanediol.

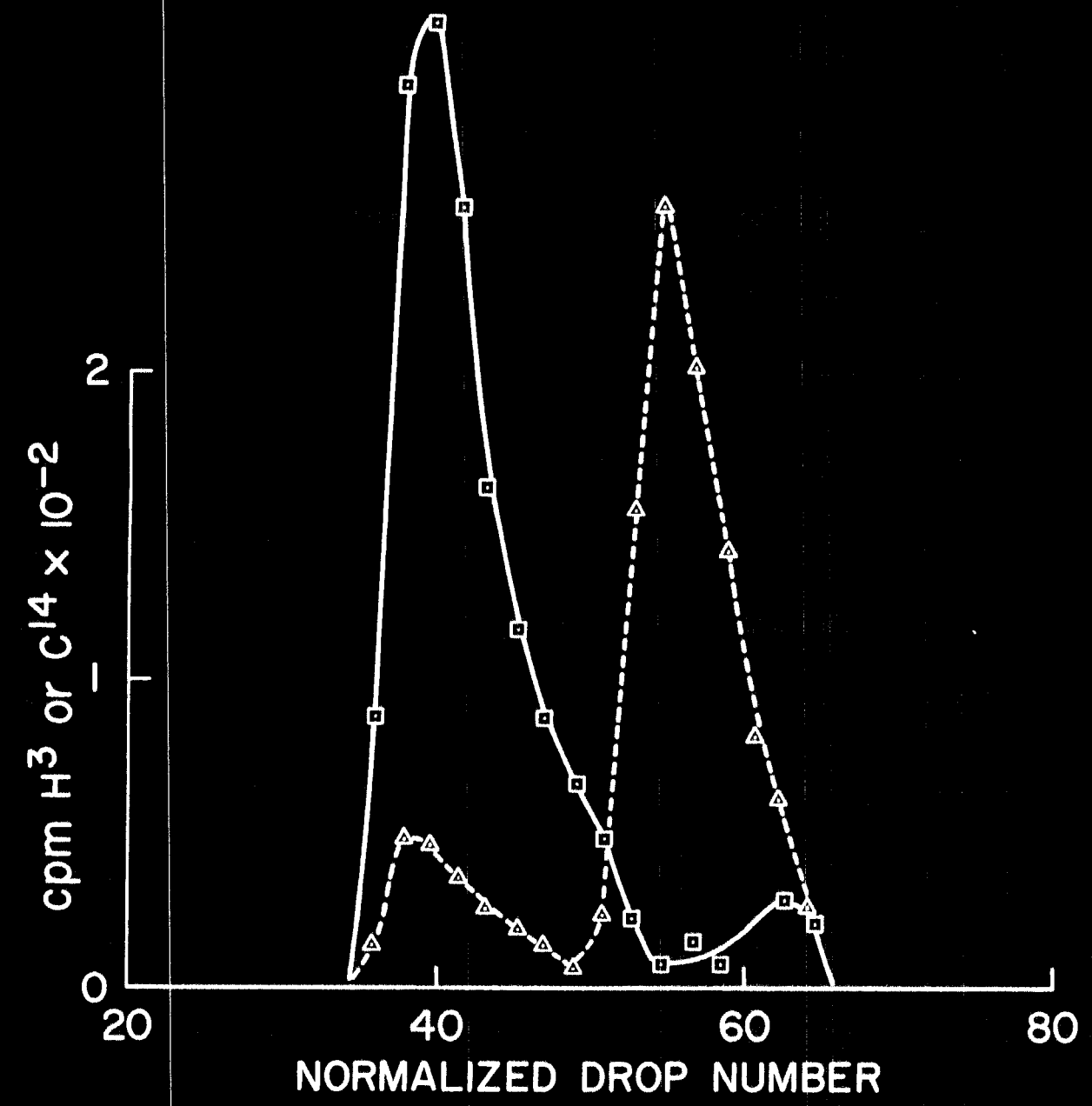

Figure 1.

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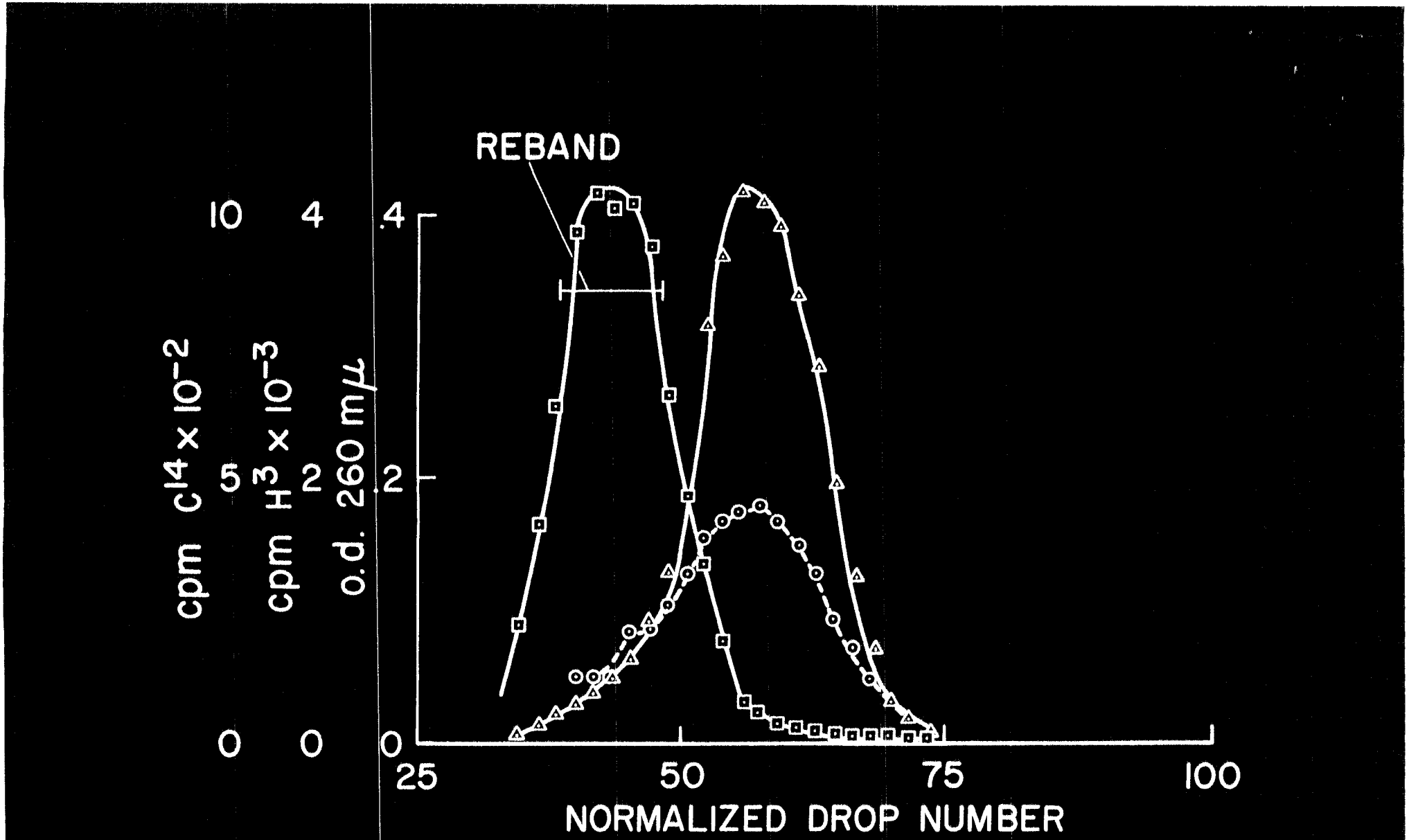

Figure 2(a).

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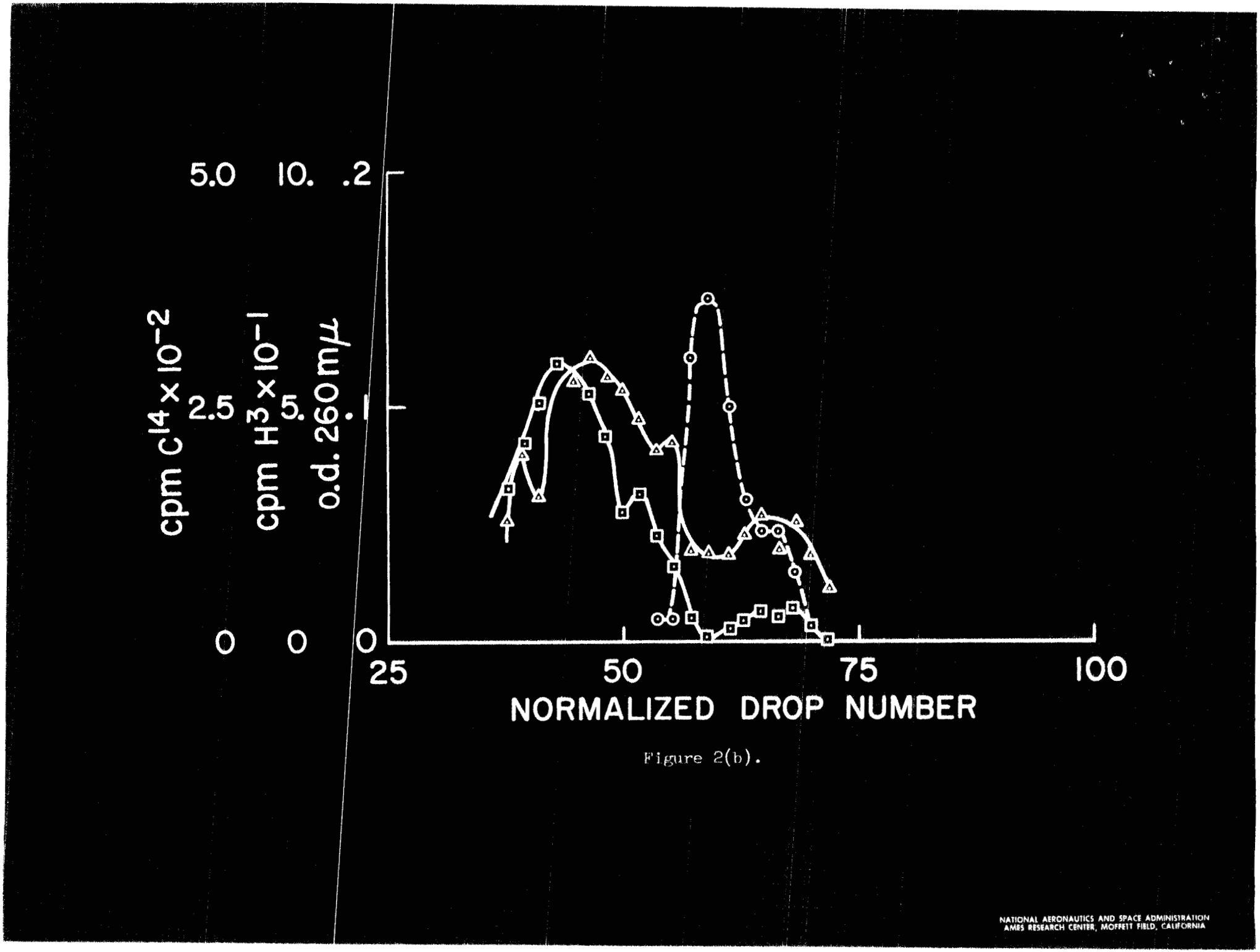

Figure 2(b).

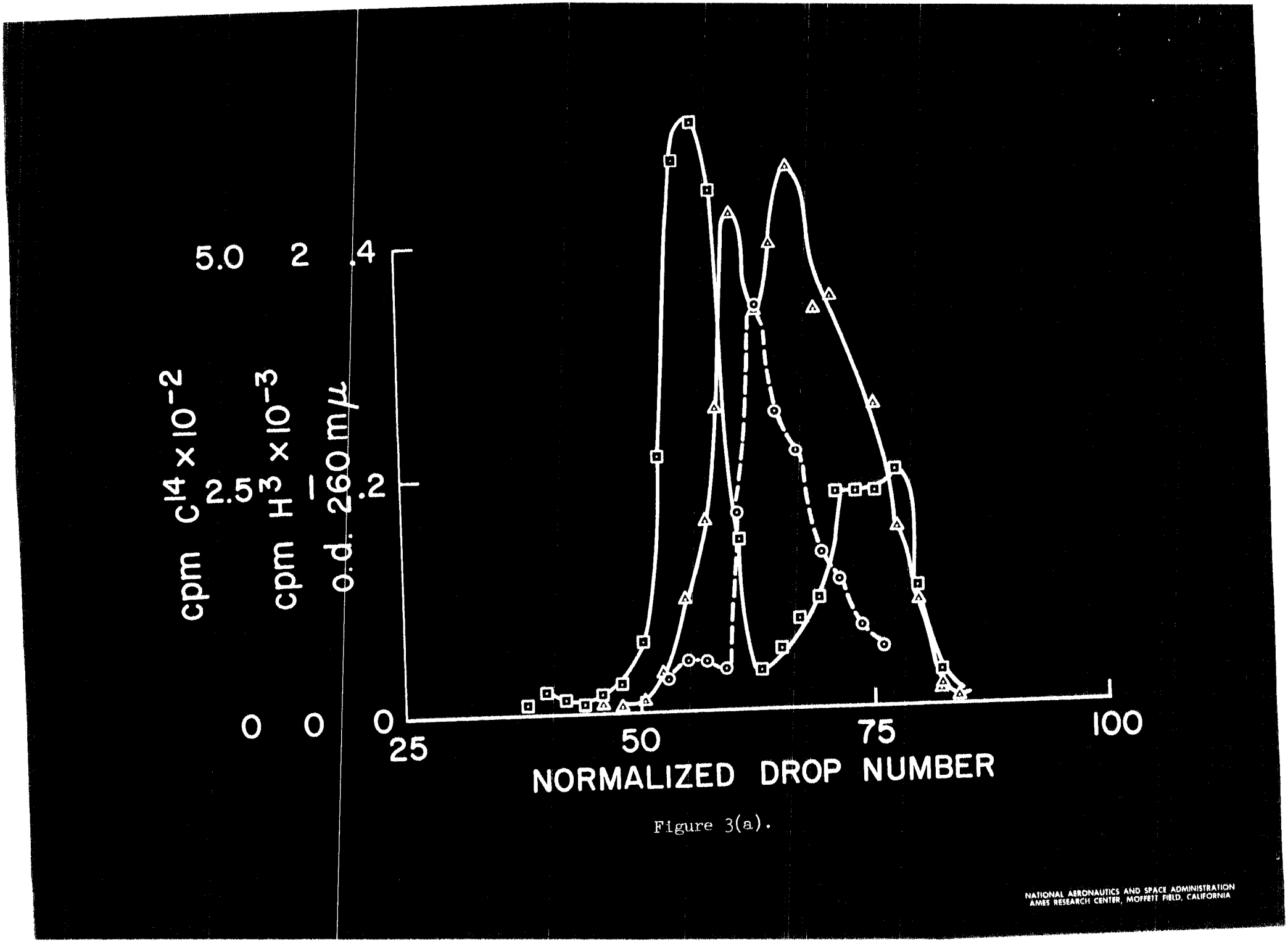

Figure 3(a).

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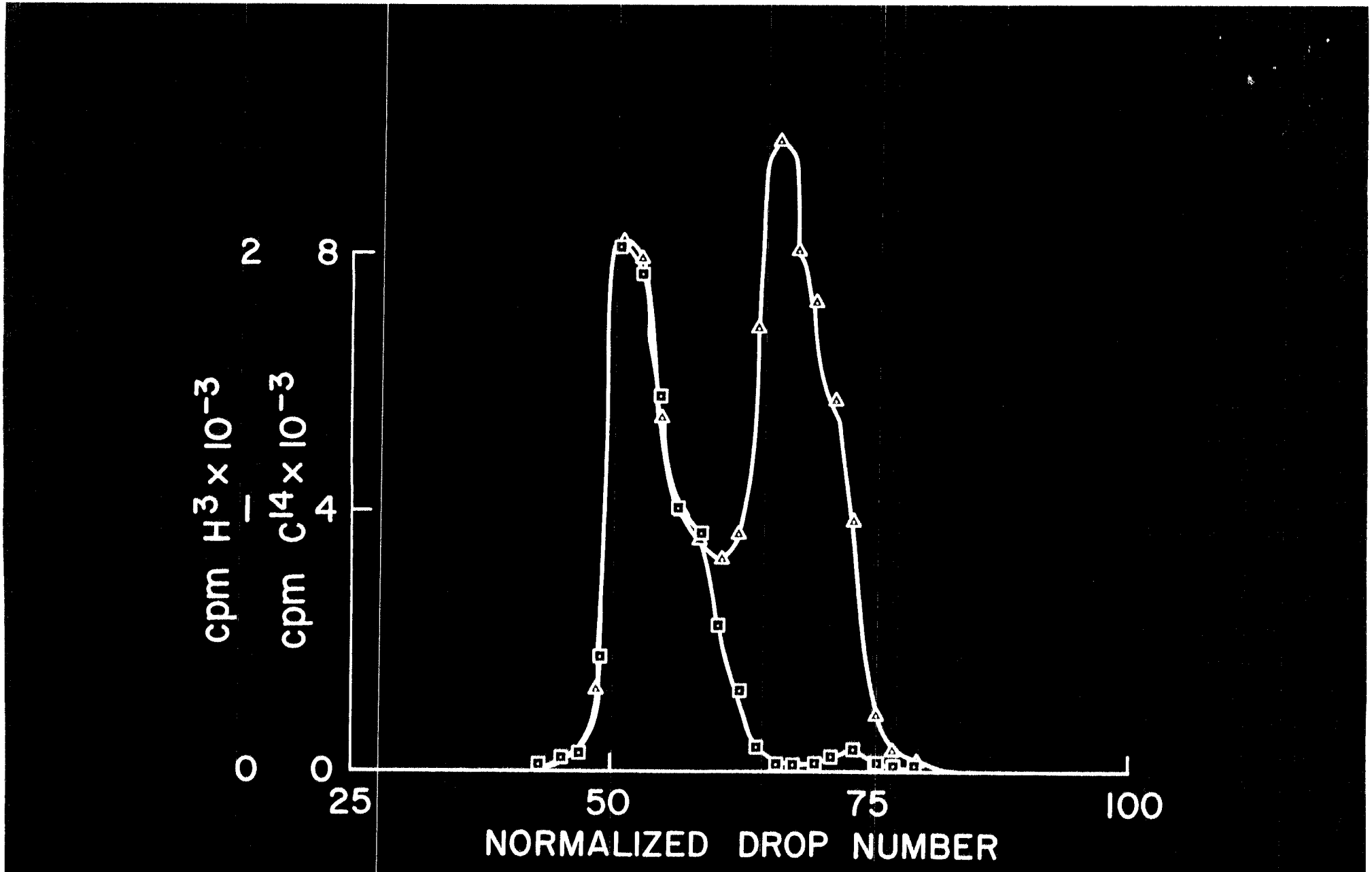

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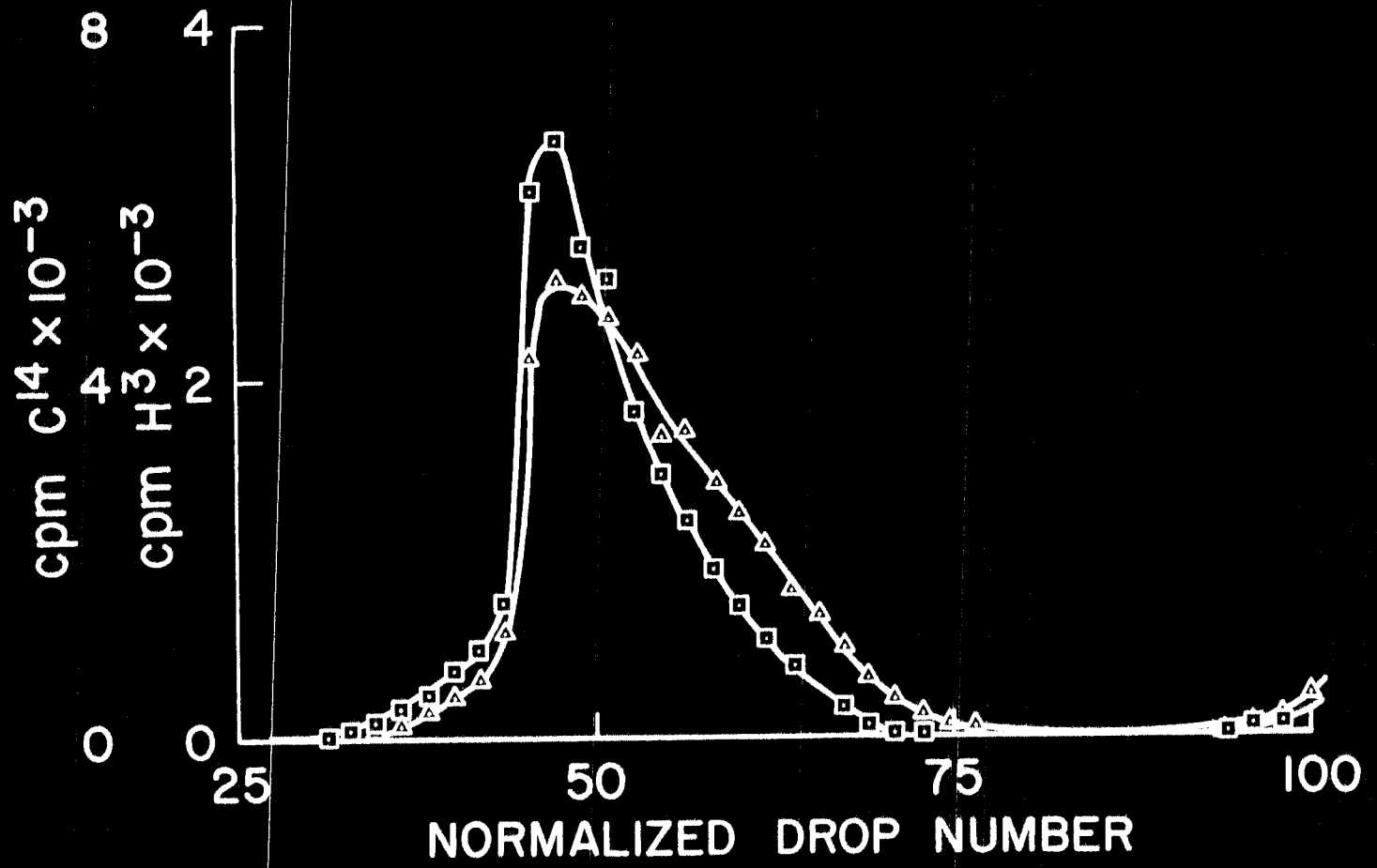

Figure 3(c)

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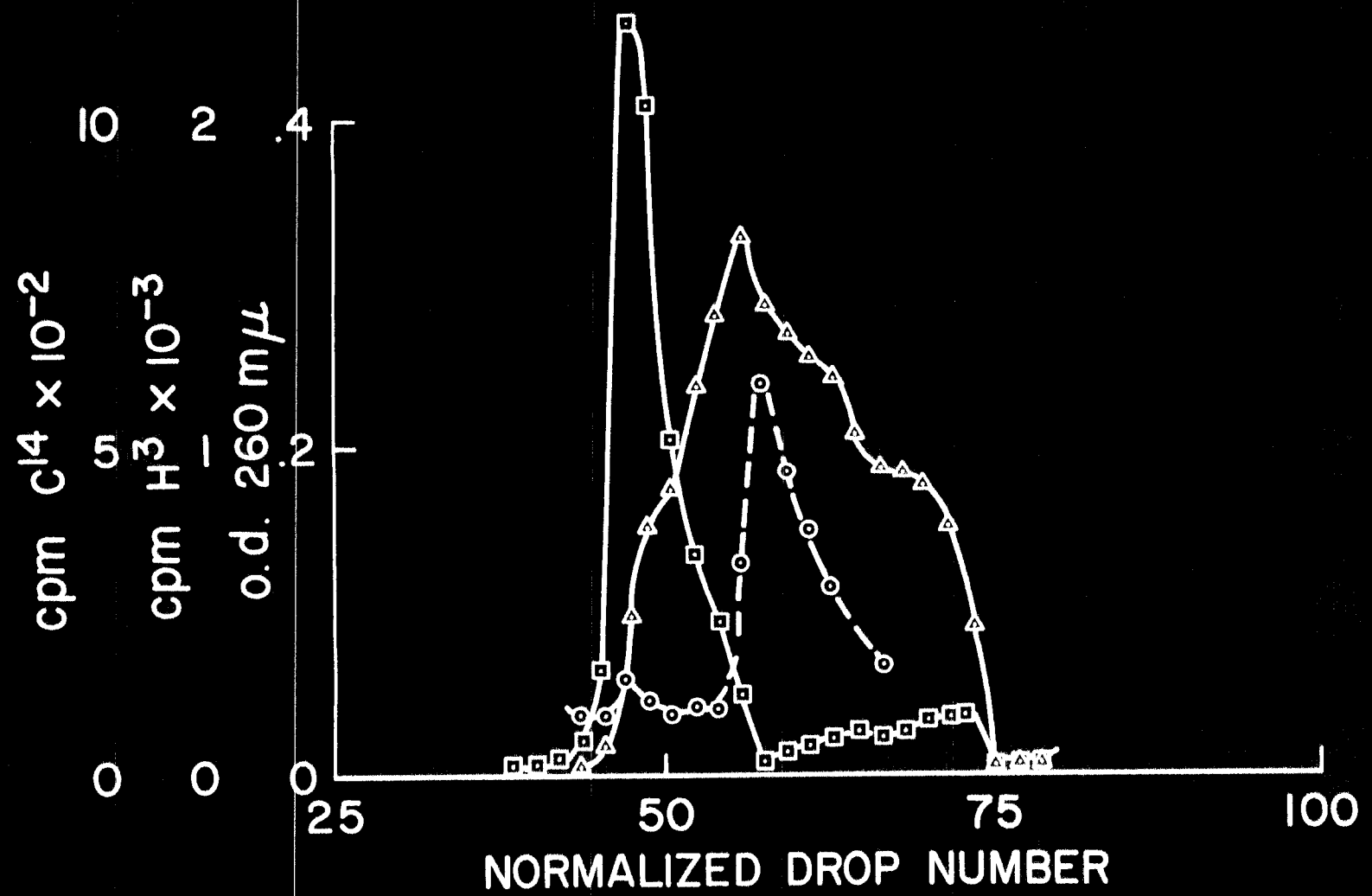

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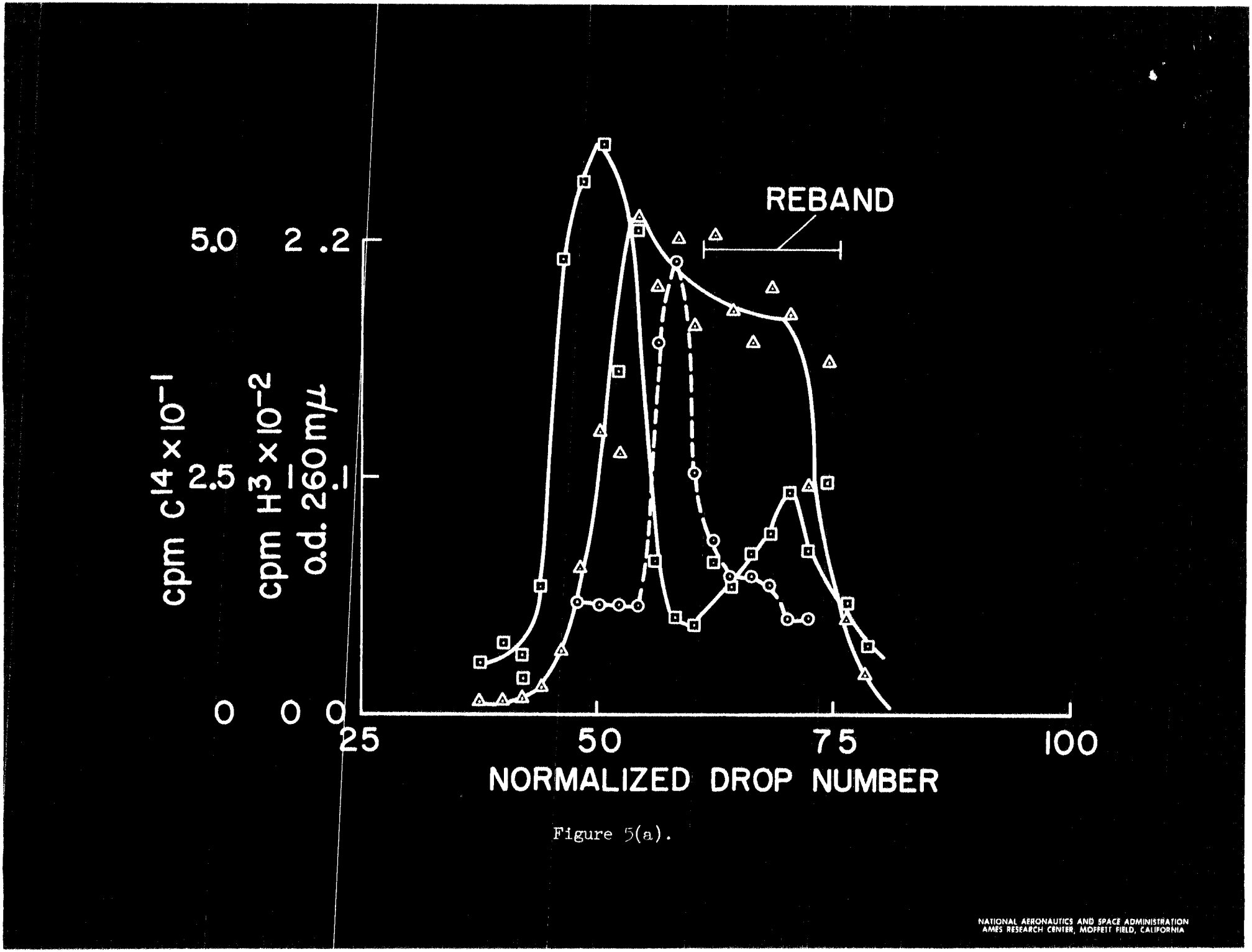

Figure 5(a).

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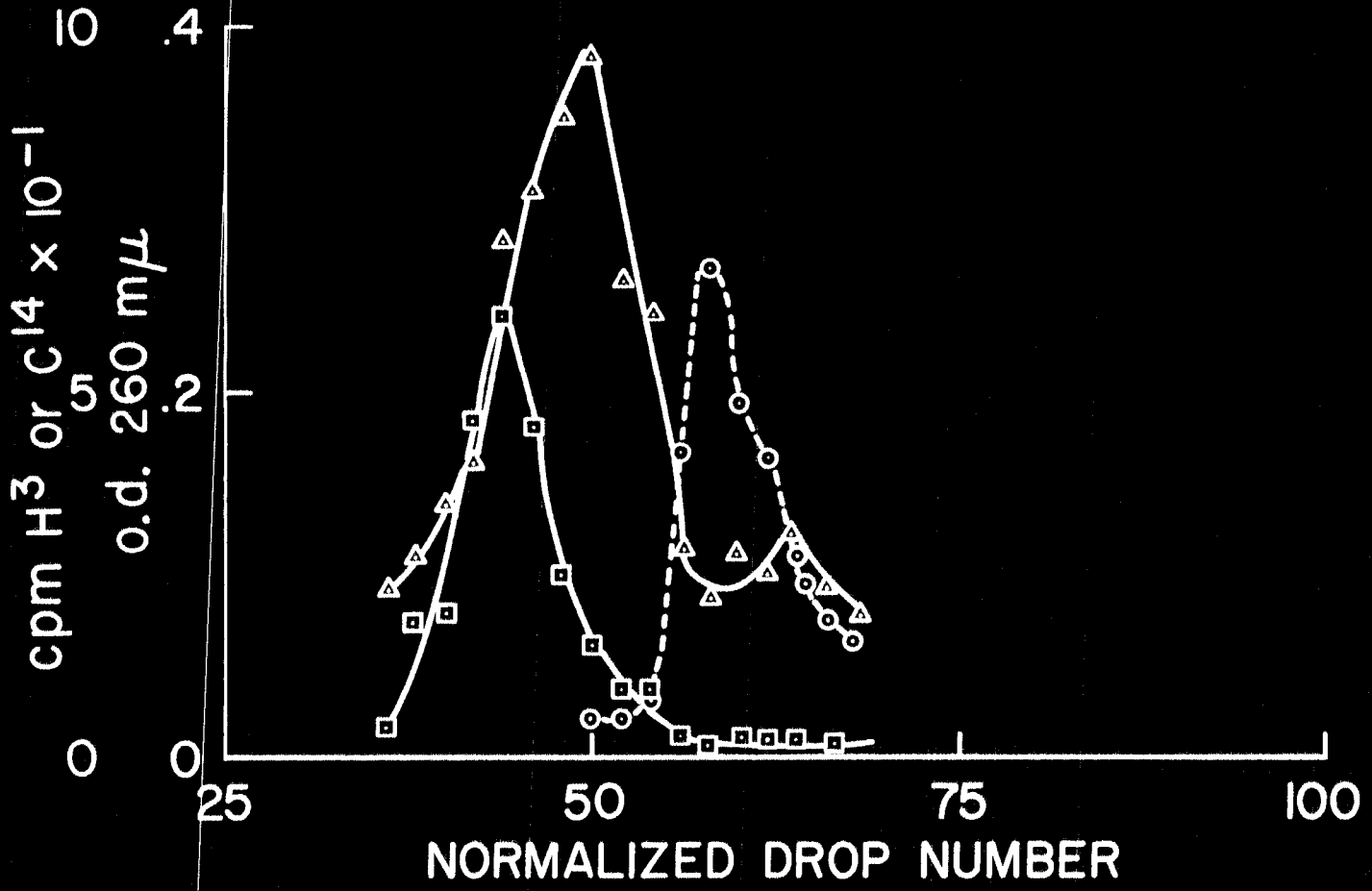

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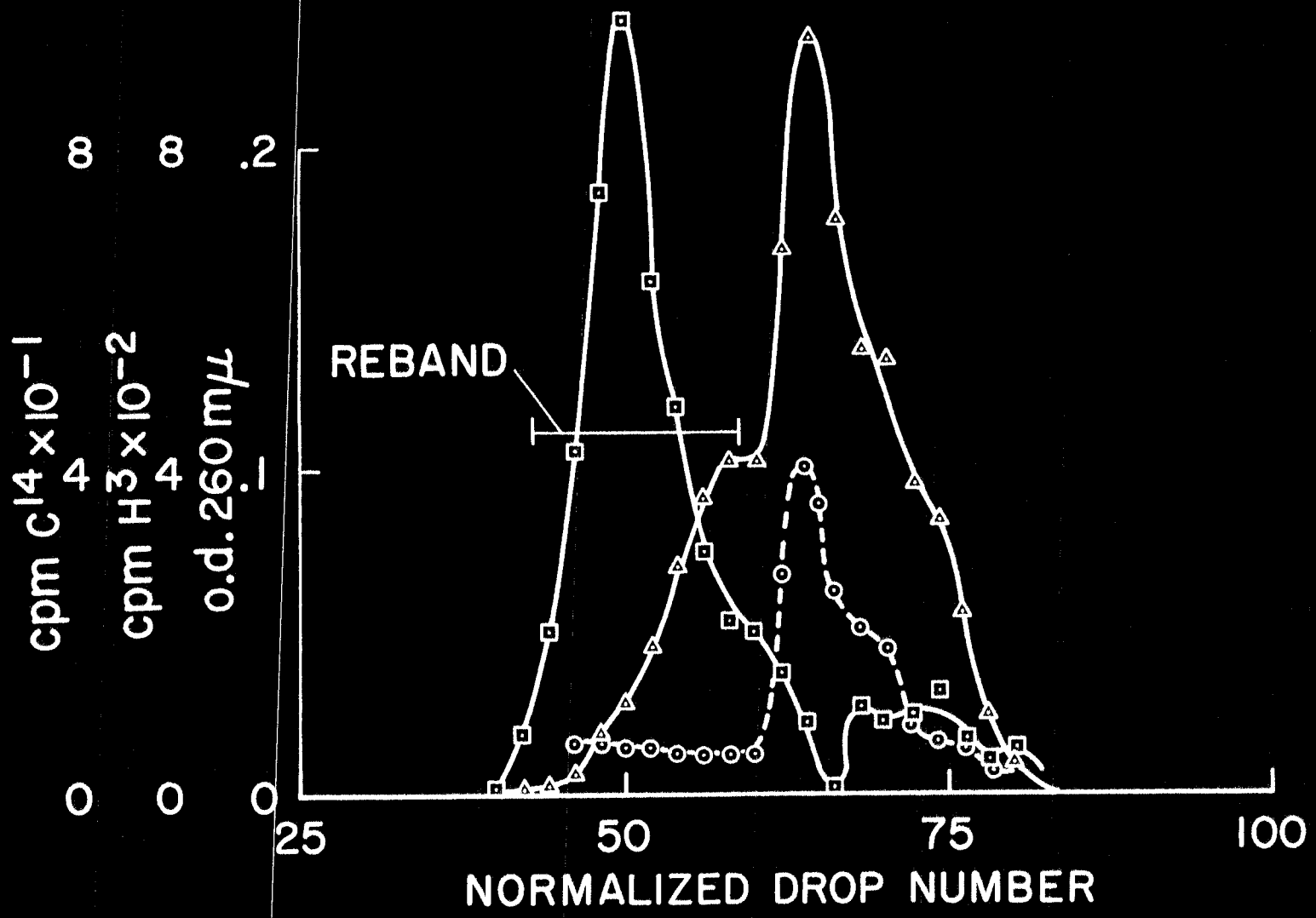

Figure 6(a).

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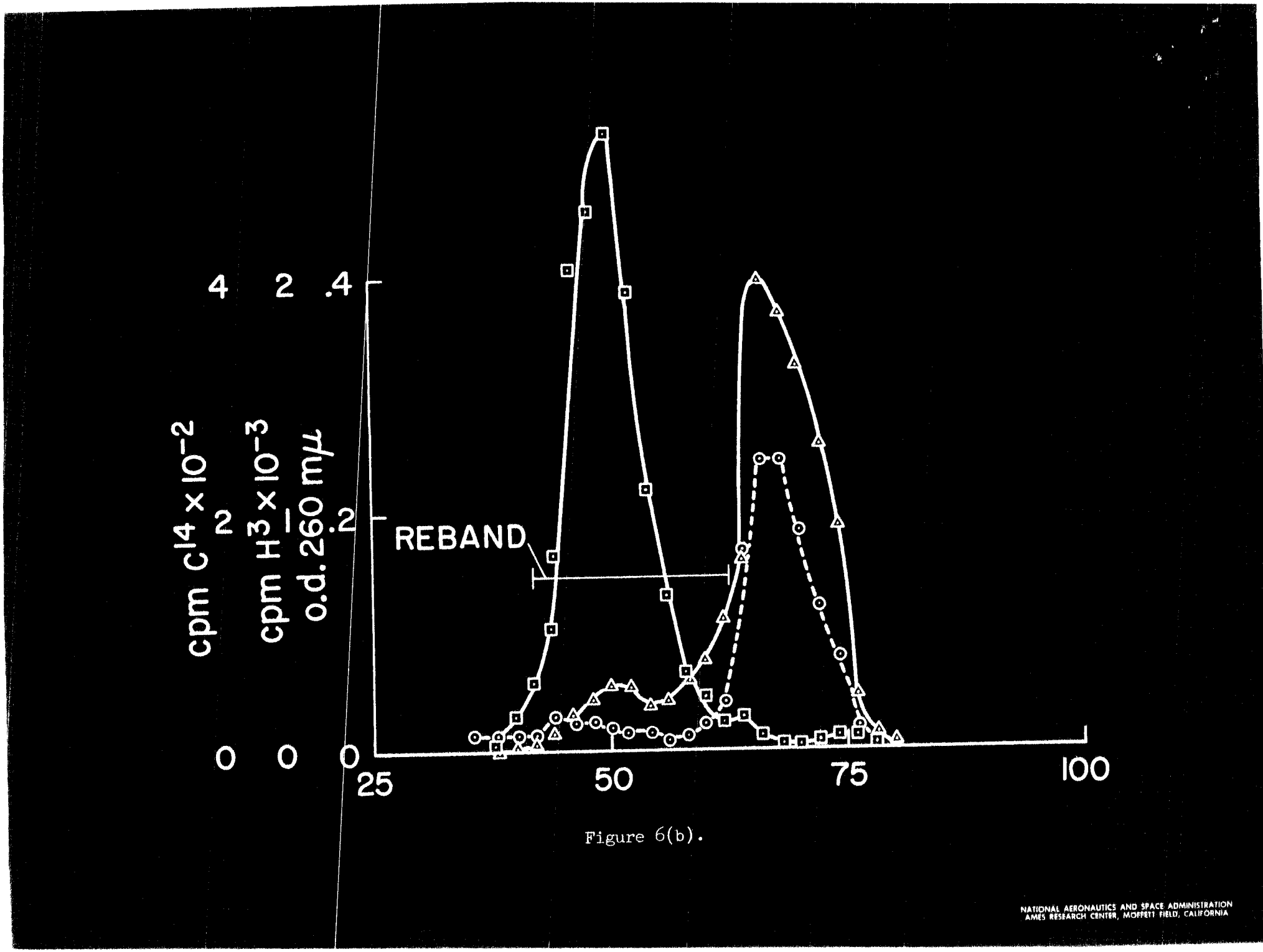

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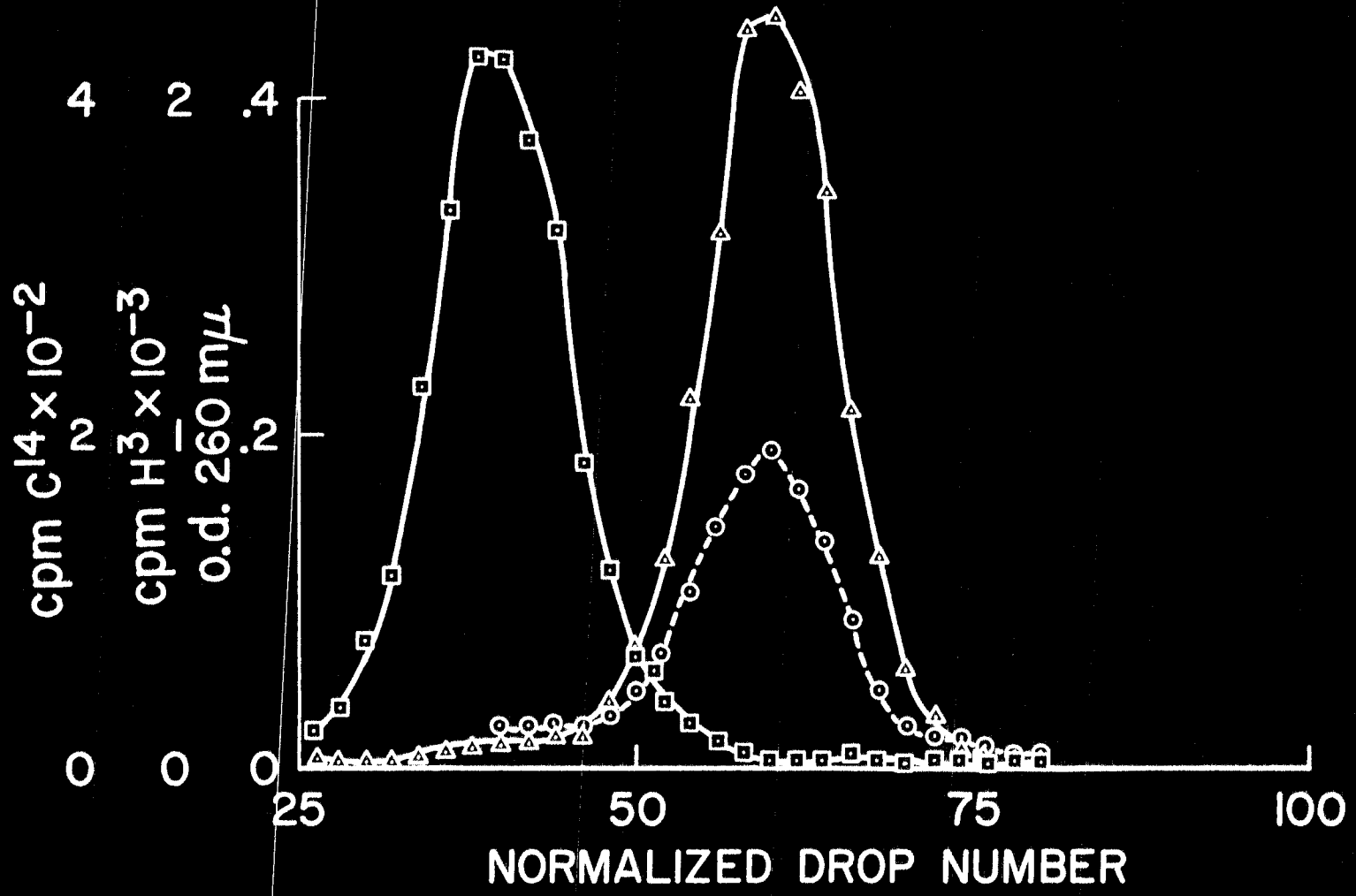

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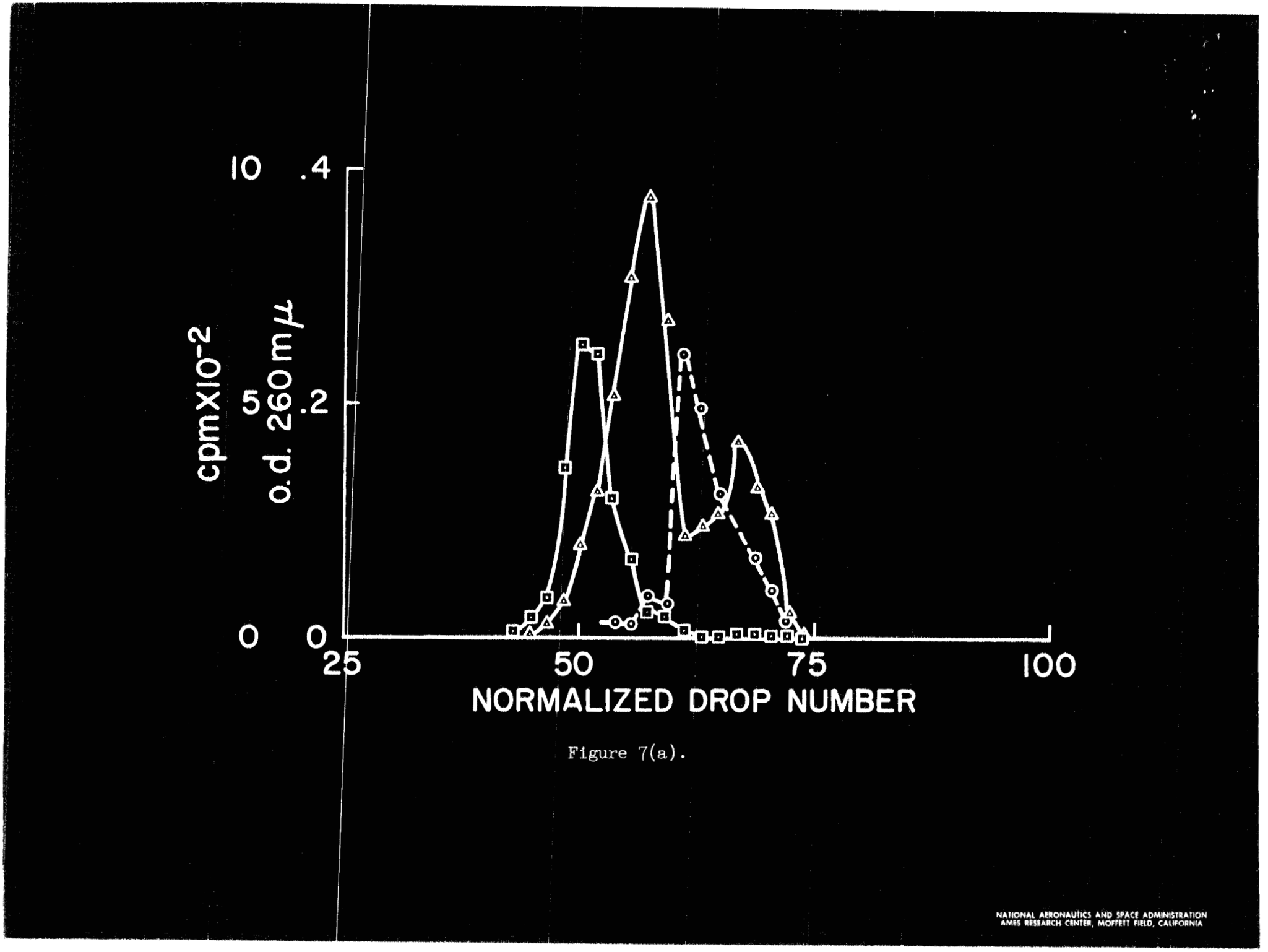

Figure 7(a).

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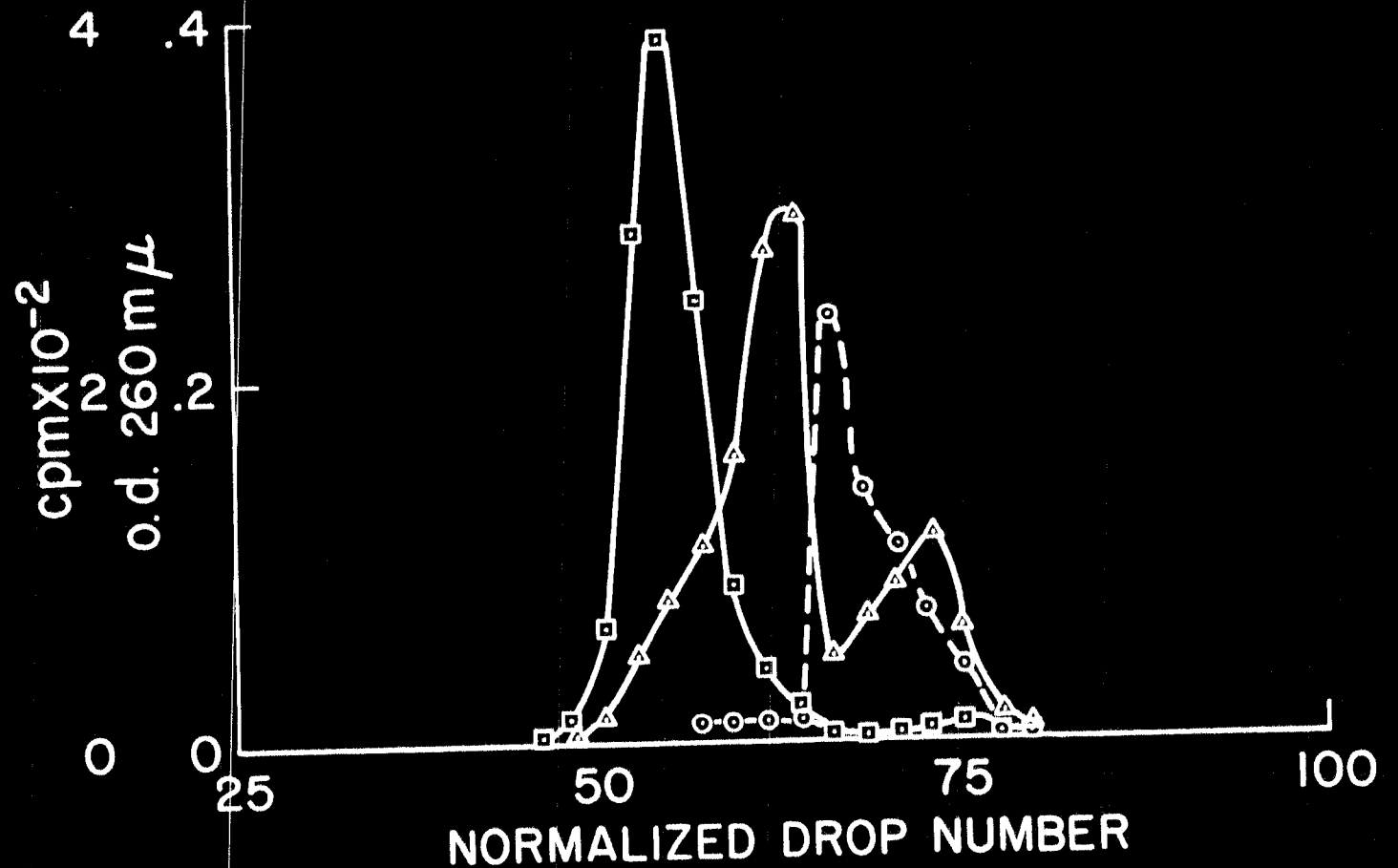

Figure 7(b).

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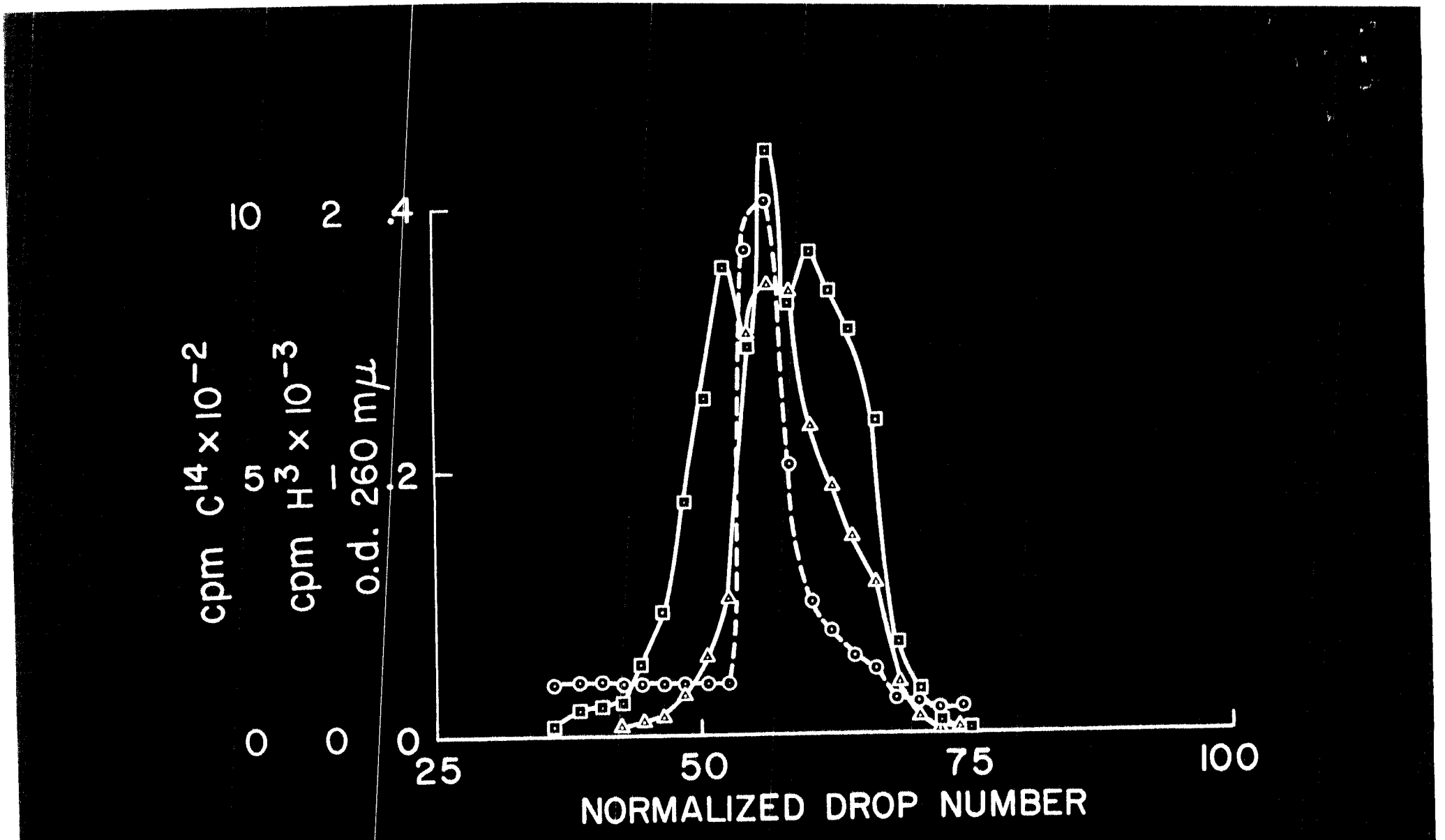

Figure 8.

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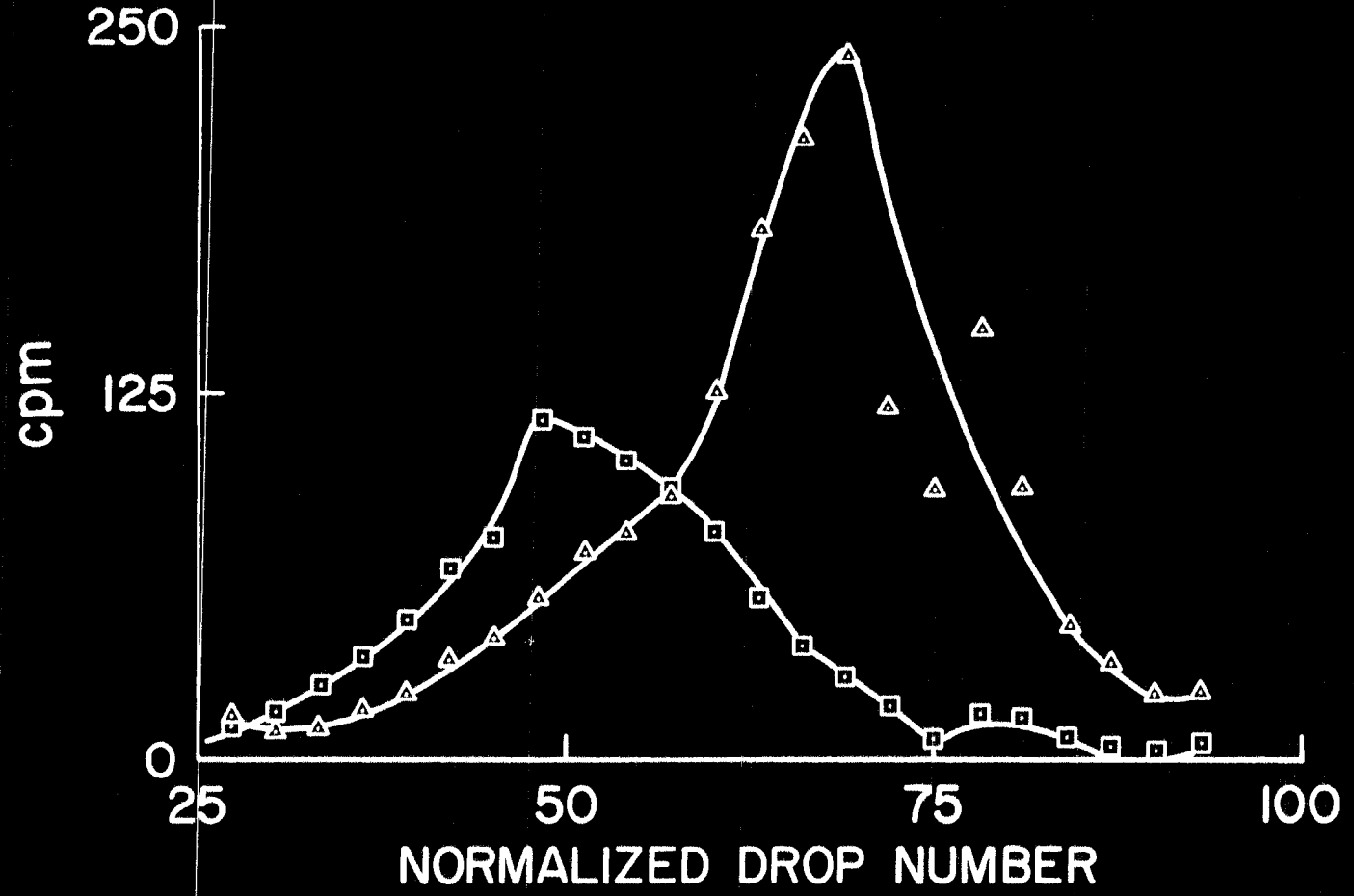

Figure 9(a).

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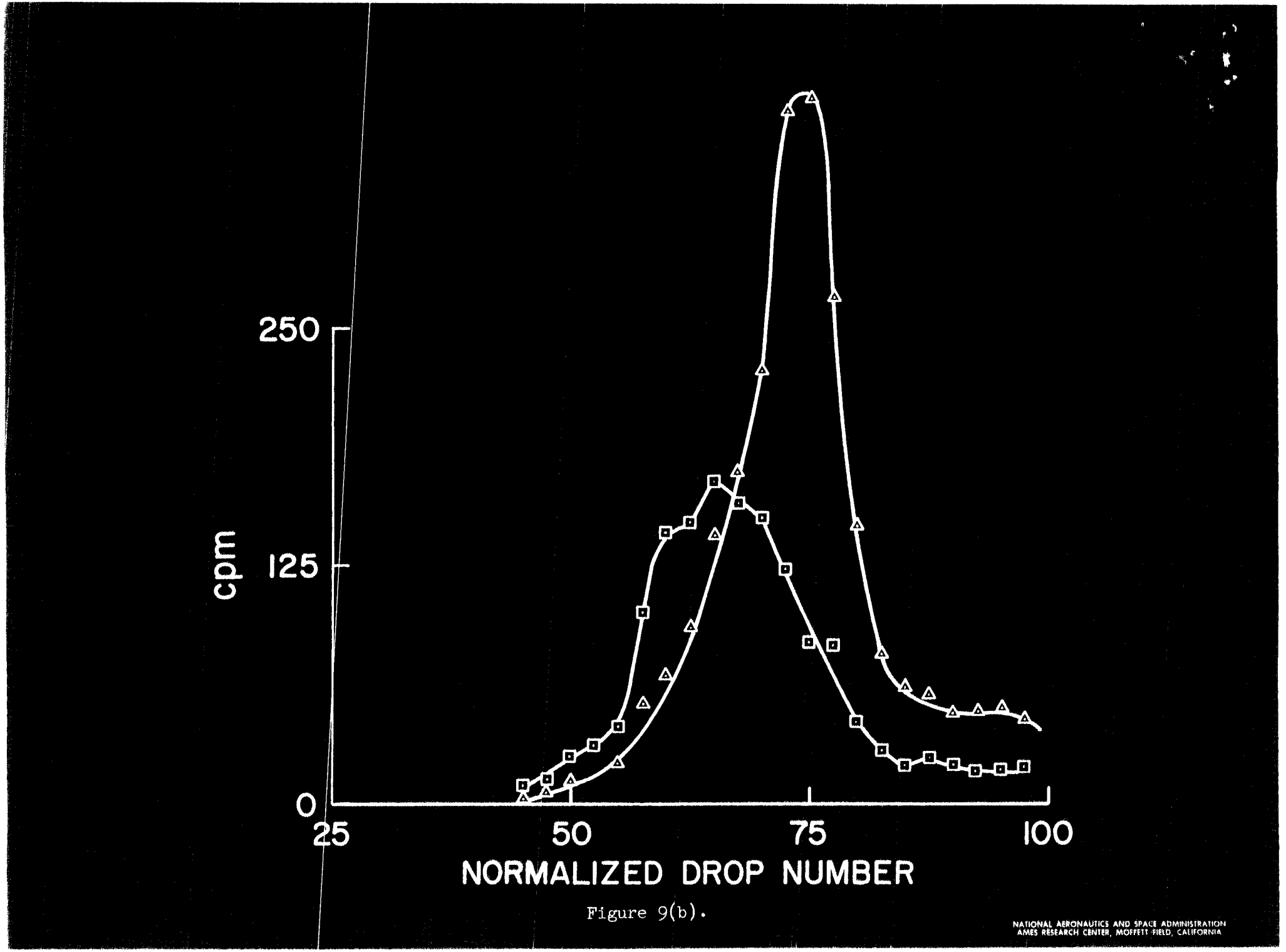

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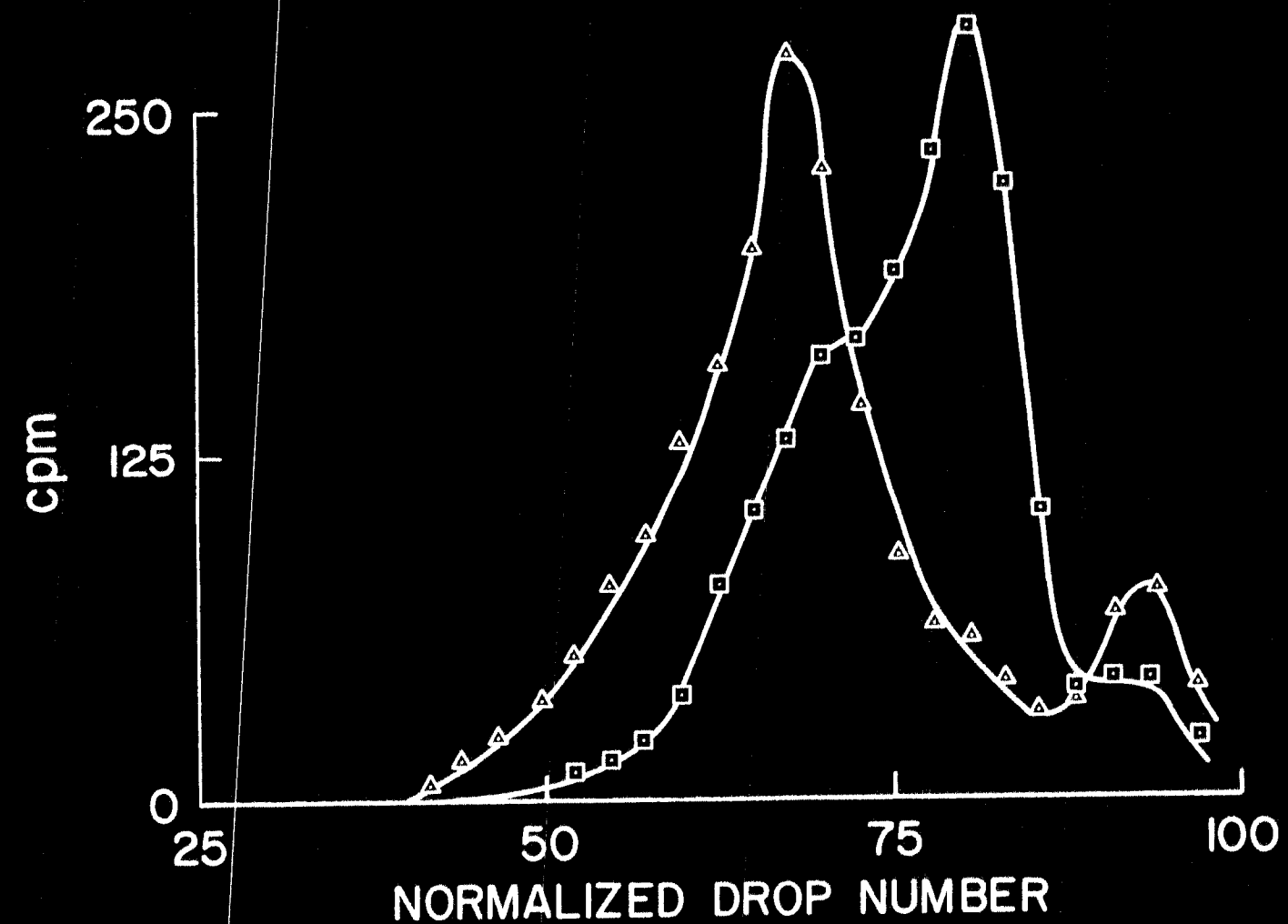

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